

Remarks

Applicant submits herewith a substitute specification in marked-up and clean copies. No new matter is introduced in the specification. The term "sylimarin" has been replaced by the correct spelling "silymarin" in the specification, as required by the Office Action.

By the foregoing amendment, claims 1-24 are amended. Entry of the amendment and favorable consideration thereof is respectfully requested.

Claim Objections

The term "sylimarin" has been replaced by the correct spelling "silymarin" in the claims as required in the Office Action.

Claim Rejection – 35 USC § 112

Claims 1-24 have been rewritten to improve clarity using a number of the Examiner's suggested amendments.

Clean copies of amended claims 1-24 are included in the clean copy of the substitute specification.

Claim Rejections - 35 USC §103

The examiner has rejected Claims 1-24 under 35 USC §103 as being unpatentable over Heidenbluth (DFD 112261-DWPI Abstract) in view of Kahol et al (US 6,399,678). The applicant respectfully traverses this finding.

The present invention provides a silymarin extraction method having improved efficiency over the prior art methods, and which does not require precooling of seeds prior to the extraction procedure.

The silymarin extraction and purification procedure described in the instant invention differs from that of Heidenbluth and of Kahol in a number of significant ways. These can best be described as follows:

Significant improvement in yield and purity of isolated silymarin

Heidenbluth reported a yield of 0.8% having a purity of 80%. Kahol et al reported a purity of 79%. The instant invention resulted in a yield of 2.0-2.5% having an overall purity of 86-97%.

Improved purity of silymarin samples results from additional interim defatting step.

In contrast to the methodology used by Heidenbluth, the defatting of the sample is carried out in two separate stages. The first defatting step is applied to the pulverized seeds. The second defatting step, which is unique to the instant invention, occurs just prior to crystallization of the final product. This second defatting step, which results in the significant improvement in the purity of the final product, is a significant inventive step and was clearly nonobvious to the inventors of the prior art.

Improved costs to manufacture and efficiency of initial defatting step.

The method of Kahol et al. requires prefreezing of the *Silybum marianum* seed material to -20 °C prior to grinding. Cooling to such low temperatures can be quite costly as it requires specialized refrigeration apparatus to reach such temperatures. More importantly, the defatting step, when carried out at lower temperatures as per Kahol, results in a substantially lower extraction of the contaminating oils than the method described in the instant invention, which describes a process of jet-milling the seed

material at room temperature. The defatting method claimed in the present invention allows for a greater extraction of the contaminating oils resulting in isolation of a final product in increased purity.

Use of nontoxic solvents

The extraction method described by Kahol requires the use of the solvents methylene chloride, methanol and acetonitrile. Each of these solvents is associated with significant toxicity. In contrast, the present invention is specifically addressed to avoid the use of such highly toxic solvents. As such, this serves as a significant improvement over the described prior art.

Conclusion

The Applicant respectfully requests the Examiner to withdraw the rejection of claims based on Heidenbluth et al. and Kahol et al. It is respectfully submitted that claims 1-24, all of the claims remaining in the application, are in order for allowance and early notice to that effect is respectfully requested.

Respectfully submitted,

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METHOD FOR ISOLATION OF SYLMARIN

[0002] ~~[0001]~~ Sylimarin is a trivial term for a complex composition of compounds: silybin, isosilybin, syldianin, sylichristin, taxifolin, and kvercetin, and its ~~the~~ most significant natural resource of sylimarin is the seeds of *Sylbum marianum*. Sylimarin has been proved as proven to be extremely hepatoprotective and it is a significant active substance in numerous herbal drugs. The most significant sylimarin component is silybin whereas the other components are present in smaller amounts. Most of the studies ~~from of~~ sylimarin pharmacology area relate to the activities of the whole composition whereas ~~whereas the individual pharmacological activities of the individual sylimarin~~ components are not well-known about. Due to this reason, the production of sylimarin is directed to as quantitative as possible the isolation from the seeds of *Silybum marianum* and further processing into a stabile crystal-like line form. Such sylimarin thus obtained is suitable for production of various pharmaceutical forms in the domains of herbal medicines or dietetics.

[0002] According to the process described in patent no. ~~U.S. Pat. No.~~ 6,309,678, the isolation of sylimarin includes cooling the seeds to -20.degree °C. ~~in order get it powdered afterwards prior to milling~~. Powdered seeds are then extracted by using n-hexane hexane as the solvent for defatting. After this, The resulting defatted seeds are further extracted with acetonitrile at room temperature. By evaporation of acetonitrile extract, a material is formed that has to be further purified by extraction with dichloromethane and the product, sylimarin, is obtained by redissolving the residue in minimal amount of acetonitrile and ~~with~~ precipitating it silymarin with by the addition of distilled water.

[0003] The dDescribed process as a part of the document includes an unacceptable precooling of the seeds to -20.degree. C. prior to defatting. Described

extracting of defatted material includes consumption of about 890 ml of acetonitrile for 100 g of the seeds (ratio 1:8.9, m/V) which is quite unacceptable for the industrial scale. Apart from this, acetonitrile is significantly toxic. By evaporation of the acetonitrile extract, such material is obtained that obviously contains some residual oil and ~~was necessarily washed~~ requires further washing with dichloromethane. Dichloromethane is a bad choice of solution for the above mentioned purpose because it is significantly toxic and its boiling point is very low, resulting in great losses on industrial scale. Purification of sylimarin silymarin by precipitating it with water from acetonitrile solution might be problematic because it can extract a product of high purity and color but resin-like consistency.

[0004] The process described in the document U.S. Pat. No. 4,368,195, just as its numerous versions, has been examined in laboratory because it was assumed that ethyl acetate was more appropriate solution than acetonitrile, due to its lower price and toxicity. The process includes defatting of the seeds by cold pressing. Such defatted residue is extracted several times (3.times.) in boiling ethyl acetate. ~~Obtained~~ The ethyl acetate extract thus obtained is evaporated and ~~its~~ the resulting residue is processed by a three-component extraction of water/methanol/dichloromethane (~~chloroform~~) wherein the residual oil passes into the dichloromethane fraction and sylimarin silymarin ~~remains~~ within the aqueous-methanolic fraction. By evaporation of the aqueous-methanolic fraction the crude product is obtained which is further purified by precipitating it from methanolic solution, by adding distilled water.

[0005] The ~~D~~described process includes cold pressing of the seeds which has its technological advantages. However, a substantial part of the oil is left in the residue so that the ethyl acetate extract contains a lot of oil, apart from sylimarin silymarin. The oil can be removed only by a three-component extraction. Furthermore, the process requires application of a large volume of ethyl acetate for the mass of the seeds, 3.times. 1:10 m/V which is ~~absolutely-non-economic for the~~ on an industrial scale. Furthermore, a three-component extraction requires the

application of a large amount of toxic organic ~~solutions~~solvents, such as dichloromethane and methanol, whose regeneration level is questionable. Furthermore, syllimarinsyllimarin lags in aqueous-methanolic fraction whose evaporation requires of which requires a relatively much-large amount of energy, wherein stability of dissolved syllimarinsyllimarin is questionable in the final phase of evaporation when the substance is exposed to relatively high temperatures in an aqueous medium, in which the solubility of oxygen from the air is by far higher than the solubility in organic solvents. Additional purification of syllimarinsyllimarin in-by way of precipitating it ~~from~~ from a methanolic solution by adding distilled water might be problematic as in the previous process because the product can be extracted in resin-like consistency.

[0006] It has been concluded that both processes are not (at least in the described form) suitable for industrial isolation of syllimarinsyllimarin from the seeds.

[0007] A new method for the isolation of syllimarinsyllimarin and oil from the seeds has been developed. The method includes grinding the seeds, extraction of oil ~~with means for~~ via a hot defatting process and extraction of defatted seeds with a medium polarity solvent. After the filtration, the extract is evaporated to dryness and the residue is azeotropically dried. ~~with adding of means for d~~ The dried Dried extract is being ~~then further~~ defatted in hot ether wherein, after the chilling, ~~filtrating~~ filtration and drying, a concentration concentrate of syllimarinsyllimarin in in the form of a homogenous yellow-orange crystal-like line substance is obtained ~~It has~~ having a high melting point (ca. 140-165.degree. C.). Yield of syllimarinsyllimarin is 2.0-2.5% according to the content of total syllimarinsyllimarin of 86-97% and approx. 20% of the oil calculated by a crude seed.

[0008] In comparison with the previously described methods, the new one process described in the instant invention does not use the toxic solvents, dichloromethane, methanol or acetonitrile. The described method applies acetone as the least toxic and by far the cheapest medium polarity solvent. The method is

developed with application ~~for the use of~~ minimal (optimum) amounts of organic solvents with a regeneration rate of approx. 90-95%. All chemical and technological details are stated in the detailed description of the method. The method comprises the following steps:

1. grinding the seeds of *Sylbum marianum*
2. extracting the seed powder with ~~as a~~ means for defatting
3. ~~filtrating~~ filtering the defatted seeds powder
4. ~~extraction~~ extraction of the defatted seeds powder with acetone
5. ~~filtrating~~ filtration of the extracted seeds powder
6. ~~evaporating~~ evaporation of the acetone extract filtrate
7. azeotropic drying of the extract with toluene
8. secondary defatting of the extract with diisopropyl ether
9. ~~filtrating~~ filtration of the resulting sylimarinsilymarin crystals
10. drying of the sylimarinsilymarin crystals under vacuum

[0009] Crude seeds of *Sylbum marianum* are powdered in using a jet mills with rotating knife ~~blades~~ with the ~~appliaction~~ application of screens up to 40 mesh; prior to ~~defatting~~. After this, defatting of the resultant seed powder is conducted with application of means for by extraction with a solvent suitable for defatting, such as a hydrocarbon. In one preferred practice of this invention, nN-hexane is used as means for ~~defatting in preferable version of the invention~~ the hydrocarbon solvent. In

another preferred practice of this invention, Petroleum ether is used in ~~other version~~ of the invention. A S~~suspension of the seed powder~~ in the applied ratio ~~herbal material~~seed powder:solvent (m/V) can normally be stirred with a mechanical stirrer.

[0010] The filtration is conducted in via vacuum filtration. The filtrate has intensive ~~intense~~ yellow color and contains the oil of the seeds in n-hexanes. The residue ~~filtered powder~~ is then heated in ~~under~~ vacuum at 70 °C for 2-3 hours to provide complete removal of extraction solvent traces and n-hexane traces. Under the stated conditions, ~~70 degree C. during 2-3 hours, there are no changes in the obtained oil if we take into account quality and quantity, i.e., there is no loss of quality.~~ According to this invention, defatted seeds do not need to be further dried in order to rid them of the traces of absorbed n-hexane but to ~~be rather~~ are extracted with a medium polarity solvent, such as acetone, immediately after vacuuming.

[0011] Extraction with acetone is conducted at ~~the a~~ temperature of approx. between 18 °C. and 56 °C. Optimum extraction time is approx. from 24 to 72 hours, depending on the temperature at which the extraction is conducted. Filtration follows the extraction. ~~The stated method provides regeneration level of acetone of approx. 95% which is, just as n-hexane in defatting phase, really being used by its 5%.~~

[0012] In the other ~~another~~ version of the invention, defatting is conducted in common percolator at room temperature during at least 48 hours. ~~In this case there is no danger of functioning at increased temperature with n-hexane:~~ After defatting and removing removal of the traces of n-hexane, percolation with acetone for isolation of sylimarins silymarin is continued.

[0013] Azeotropic distillation follows, by which water and toluene is removed from the residue after evaporating ~~evaporation of~~ the acetone filtrate is removed. During this, after evaporating ~~After evaporation of~~ the acetone in the filtrate, ~~a~~ receiving plate is replaced, wherein the a mixture of toluene and water is collected ~~gathered~~. The stated toluene and water mixture is easily separated in an

~~the extractor wherein the upper, toluene fraction is stored. Under the stated conditions, and 80% of the used toluene is regenerated regenerated with a part of it being wasted in the vacuum system. However, work in A vacuum system is used is necessary to provide drying at a temperature as low as possible.~~

[0014] For purification, i.e. removing the residual oil, according to the invention ethers are applied as a means of extraction. According to the invention, ethers with 4 to 8 carbon atoms such as tetrahydrofuran, or diisopropyl ether, or diethyl ether are appropriate for secondary defatting.

[0015] It has been found that diisopropyl ether acts as the most efficient solvent for defatting the extracts wherein the oil is completely dissolved while ~~syllimarin~~syllimarin constituents remain almost completely suspended. One part of the evaporated residue can stay attached to the walls of the ~~extraction flask~~ and it is not removed spontaneously during heating at the reflux temperature ~~for the return of the solvent~~. Therefore, ~~a mechanical intervention~~ stirring is needed. On an industrial scale, a reactor with mechanical mixer whose shape follows the geometry of the extraction flask is used. The purpose of it is to avoid the ~~syllimarin~~syllimarin getting glued to the walls ~~as seen as during~~ azeotropic drying with toluene. Once formed, the suspension of ~~syllimarin~~syllimarin constituents is nicely defatted and easily filtered in vacuo after the cooling. Further cooling of the suspension does not substantially ~~a~~affect the level of seproduct purity, since the solubility of ~~syllimarin~~syllimarin constituents in diisopropyl ether is extremely low. The next example serves only as an illustration of the invention and ~~can by no means is~~ is not meant to be used for defining the range and content of the invention.

THE EXAMPLE

[0016] 400.00 g of ~~grinded~~ground *Sylbum marianum* seeds were weighed in a 2000 ml three-necked flask of 2000 ml and 1200 ml of n-hexane was poured into it ~~added~~ (of approx. 96%, Merck, for synthesis). ~~Obtained~~ The suspension thus obtained

was heated with stirring with using a mechanical mixer and heated to reflux ; during 30 minutes. The refluxing suspension was stirred at the temperature of the return of solvent, approx. 62-63.degree. C., during for an additional 3 hours. After this, the mixture was cooled cooling, the herbal material sucked out through the vacuum filtered using a Buchner funnel with subpressure. 2.times.100 ml of n-hexane was used for the removing and washing of The flask was then washed with n-hexane (2 X 100 ml) while additional 2.times.100 ml of n-hexane was used for washing of the sucked out herbal material. The remaining seed powder was further washed with n-hexane (2 X 100 ml). Approx. 1500 ml (about 95% of the whole amount of the used n-hexane) of n-hexane was pre-distilled distilled from the filtrate and thus regenerated from the filtrate while the leaving a residual pale yellow oil which was heated at 70.degree. C. in vacuum, at 8-10 mbar, during 2 hours to remove any remaining traces of hexane. A cClear yellow-orange oil of weak characteristic scent was obtained:

[0017] BATCH-1: 60.05 g (15.0%, calculated by weight of the a-crude seed)

[0018] BATCH-2: 61.41 g (15.4%, calculated by a-weight of the crude seed)

[0019] BATCH-3: 61.98 g (15.5%, calculated by a-weight of the crude seed) The dDefatted Sylbum marianum seeds (approx. 340 g) were then removed into the 2000 ml three-necked flask of 2000-ml and 1200 ml of acetone was poured into it, and the obtained suspension was being stirred with a mechanical mixer at the room temperature during 72 hours. After this, the suspended herbal material was sucked out vacuum, filtered through the Buchner funnel with subpressure. For removal of the residual herbal material, 2.times.100 ml of acetone was used, and 2.times.100 ml of acetone for additional washing of herbal material. The filtered material was then twice washed, each time with two 100 ml portions of acetone. From the obtained yellow filtrate (approx. 1550-1580 ml), acetone was pre-distilled removed by distillation at the atmospheric pressure, wherein approx. 1500-1530 ml (93.8-95.6%) of the whole amount of it was regenerated.

[0020] After removal of the acetone was complete, 60 ml of toluene was added to the residual and the resulting solution was evaporated in a rotating evaporator at the a temperature of 80-85 °C and the a pressure of 50-60 bar, wherein 25-27 g (6.25-6.75 g) of dry extract was obtained in form of yellow-orange crystals, mottled with yellow-orange oil. 50 ml of diisopropyl ether was added to the dry extract and with stirring heated to the temperature at which the solvent returnsrefluxed, approx. 67-69 °C., during 25-30 minutes. The suspension was heated with stirring in hot diisopropyl ether during the next 30 minutesHeating was continued for an additional 30 min. after which. After this, the suspension was allowed to cooled to the room temperature during approx. 1 hour. The resulting cCrystals were then sucked-out-filtered and washed with 2.times with 25 ml od portions of cold diisopropyl ether. The product was dried in a vacuum drier at 40 °C. and the pressure of 8-10 mbar, during 24 hours. SylimarinSilymarin (1), in form of small shiny crystals, with the color ranging from yellow to light orange, was obtained.

[0021] BATCH-1: 8.52 g (2.13%), t. t. 142.2-165.0 °C [0022] BATCH-2: 9.02 g (2.26%), t. t. 143.0-164.2 °C [0023] BATCH-3: 8.91 g (2.23%), t. t. 140.2-161.1 °C

From the yellow diisopropyl ether filtrate, after the filtration of sylimarinsilymarin (approx. 100 ml) by distillation at the atmospheric pressure, 95-96 ml (95-96%) of diisopropyl ether was regenerated. The residue was dried in vacuum at 8-10 mbar with heating at 70 °C., during 2 hours wherein a secondary batch of yellow-orange oil was obtained. The oil was to be further filtrated-filtered to be rid of traces (less than 1%) of insoluble material suspended in the diisopropyl ether of soluble compounds from the extract.

[0024] BATCH-1: 17.84 g (4.46%, calculated by a weight from the crude seed)

[0025] BATCH-2: 17.23 g (4.31%, calculated by a weight from the crude seed)

[0026] BATCH-3: 18.70 g (4.68%, calculated by a weight from the crude seed)

The total of obtained oil: (primary+secondary)

[0027] BATCH-1: 77.89 g (19.5%, calculated by a weight from the crude seed)

[0028] BATCH-2: 78.64 g (19.7%, calculated by a weight from the crude seed)

[0029] BATCH-3: 80.86 g (20.2%, calculated by a weight from the crude seed)

Optimum conditions of drying of sylimarinsilymarin have been stated. Due to its phenolic components, sylimarinsilymarin is sensitive to oxygen and light. The stated method enables successful drying of sylimarinsilymarin because after it is sucked outfiltered, it comprises-contains only trace amounts of absorbed diisopropyl ether from which it is very easily dried.

[0030] Diisopropyl ether, acetone and n-hexane are regenerated to approx. 95% of their use and can be used again for the same purpose reused. Commercial diisopropyl ether is stabilized with approx. 50 ppm of antioxidant, such as 2,6-di-tert-butyl-4-methylphenol (BHT), however, solvent which is regenerated after distillation will do not contain a stabilizer any more. In case of frequently repeated application of the same diisopropyl ether, solvent as such can be used without problems stored for short periods of time with a short storing in well sealed barrels. For longer storage periods, storing-BHT (the cheap one) is to be added to the regenerated solvent, approx. 5 g ea-per 100 l.

SylimarinSilymarin obtained by the described method is a crystal-like matter of orange colour, without scent, and is melted-melts at a temperature range between 140 and 165.degree. C.

In sylimarinsilymarin IR-spectrum, a characteristic stretching band of alcoholic and phenol O-H bonds at 3400, and ketone stretching band of chetonic C.dbd.O bond of at about 1640 cm are visible.

[0031] IR (KBr) v: 3401 (O-H, phenol group), 2928, 1745, 1641 (C.dbd.O, keto-group), 1513, 1465, 1358, 1275, 1160, 1160, 1083, 1027, 992, 813, 782, 644

cm.

In IR spectrum of Silybum marianum oil the band appears at about 1744 cm. It is characteristic for stretching of esteric C=O bond an ester carbonyl.

[0032] IR (film) v: 3009, 2926, 2855, 1744 (C=O, esteric ester group), 1656, 1466, 1418, 1378, 1239, 1163, 1099, 914, 723 cm.

Qualitative (IR spectrum, thin-layer chromatography) and quantitative (spectrophotometry) analysis were conducted on the obtained syllimarin.

[0033] Quantitative analysis of syllimarin concentrate obtained by the described method was done in spectrophotometry way spectrophotometrically with the use of 2,4-dinitrophenylhydrazine (DNPH) as reagent, by the method described in the literature (H. Wagner, P. Diesel, M. Seitz, Arzneim. Forsch. (Drug Res.) 24 (1974) 466-471). The results are in Table 1. TABLE-US-00001 TABLE 1 The results of quantity content of the total syllimarin in syllimarin samples, batches 1, 2 and 3, obtained according to the described method. Calculated absorption measured measured-per SYLIM. TOTAL SAMPLE weight absorption 50.00 mg. subs. (%).sup.1 Silibyn-stand.sup.2 0.05044 0.237 0.235 100 Syllimarin, B-1 0.05043 0.214 0.212 90.21 Syllimarin, B-2 0.05002 0.203 0.203 86.38 Syllimarin, B-3 0.05010 0.227 0.227 96.60 .sup.1The content of total syllimarin presents the content of total ketone, i.e., all the syllimarin components with ketonic ketone functionality (so called DNPH of positive compounds). .sup.2_The silibyn standard of sylimarin prepared by preparatory chromatography, was declared as 100% syllimarin.

Literature

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ABSTRACT

~~This invention relates to a method for isolation of silymarin from silybum marianum seeds wherein the seeds are grinded without precooling. The follow defatting with hydrocarbon solvent, extraction with medium polarity solvent, removal of water from evaporating residue lagged after extraction, purification of dry extract, separation, washing and drying of obtained crystals~~This invention relates to a method for the isolation of silymarin from *silybum marianum* seeds without precooling, wherein the ground seeds are defatted using a hydrocarbon solvent, followed by extraction with a nontoxic medium polarity solvent, purification of the dried extract and drying of the crystals thus obtained.